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TITLE: Sildenafil and phosphodiesterase-5 inhibitors to reduce cardiotoxicity and enhance the response of breast tumors to doxorubicin

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14. ABSTRACT: The overall goal of this work is to determine the basis for the differential effects of phosphodiesterase inhibitors, such as sildenafil, in terms of protecting cardiac cells and the heart from the toxicity of the antitumor drug adriamycin, while failing to protect the breast tumor cell. In the current work, we have substantiated our previous observations in the breast tumor cell and extended these findings to other chemotherapeutic drugs (taxol and cisplatin) as well as ionizing radiation. However, we have been unable to demonstrate protection from adriamycin in a different model of cardiomyocytes. This may relate to a number of factors that are currently under investigation, including the high concentration of adriamycin used for a prolonged time period as well as the possible absence of phosphodiesterase-5 as a target in these cells. We believe that a more extensive analysis of the nature of the response to sildenafil in these cardiomyocytes will provide insights as to the mechanism(s) of cytoprotection.

15. SUBJECT TERMS
sildenafil; breast tumor; cardiomyocytes; cardiomyopathy; adriamycin; chemotherapy; selectivity; protection; apoptosis

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INTRODUCTION

Our proposed work was based on the observations that phosphodiesterase 5 inhibitors, specifically the erectile dysfunction drugs such as sildenafil, could protect cardiomyocytes and the heart from the toxicity of the anthracycline antibiotic, adriamycin (1). Furthermore, we had generated preliminary data that sildenafil did not appear to protect the breast tumor cell from adriamycin and, in fact, may have promoted sensitivity to this drug. Our research goals were to extend these observations and to delineate the mechanistic basis for these differential effects in the tumor and in the heart.

BODY

We have repeated some of our original studies using MCF-7 (p53 wild type) and MDA (p53 mutant) breast tumor cells and have verified that sildenafil *does not* protect breast tumor cells from adriamycin (Figures 1 and 2); furthermore, in some experiments, sildenafil did, in fact, appear to make the cells slightly more sensitive to this drug treatment (data not shown).

In view of the fact that patients are rarely treated with a single drug such as adriamycin, but are generally exposed to multiple drugs of different mechanisms of action as well as irradiation therapy, it was important to substantiate that sildenafil did not interfere with other chemotherapeutic treatments. To this end, the cells were treated with (individually) camptothecin, taxol, and cisplatin as well as ionizing radiation both in the absence and presence of sildenafil. As shown in Figures 3, studies in MDA-MB231 breast tumor cells confirmed that sildenafil fails to protect breast tumor cells from a number of the drugs routinely used in the treatment of breast cancer or from ionizing radiation. Similar data were generated in MCF-7 cells (not shown).

We also performed a series of studies assessing the capacity of sildenafil to protect MCF-7 cells expressing the executioner caspase, caspase 3, from adriamycin. Caspase 3 is frequently considered to be critical for the promotion of apoptotic cell death in experimental model systems, while MCF-7 cells do not express this caspase (2). There is also uncertainly in the scientific literature as to whether clinical samples of breast cancer express caspase 3 (3). Figure 4 indicates that sildnafil also increased sensitivity to adriamycin in this cell line over a range of concentrations of adriamycin. These findings support the fundamental hypothesis underlying this grant, that sildenafil will not be protective to breast tumor cells treated with adriamycin.

Our previous work, which generated the preliminary data presented in our proposal, evaluated the interactions between sildenafil and adriamycin using simple assays of viable cell numbers. However, the gold standard for assessment of drug and radiation sensitivity is clonogenic survival. To this end, we evaluated the effects of sildenafil on the reduction of clonogenic survival in response to adriamycin in MCF-7 cells, MCF-7/caspase 3 cells and MDA–MB231 cells. As shown in Figure 5, sildenafil enhanced the sensitivity of these tumor cells to adriamycin, supporting the results of our preliminary work.

In terms of exploring the mechanisms whereby sildenafil might protect only cardiomyocytes while allowing adriamycin to target the breast tumor cell, it is important to recognize that the foundation for differential effects is likely to doxorubicin acting as a topoisomerase II poison in

tumor cells while its toxicity to the heart is through the generation of free radicals. (4). It should be noted, however, that there is also data suggesting a free radical mechanism of action for adriamycin in the breast tumor cell (5). We assessed whether sildenafil could protect against hydrogen peroxide, which is toxic through the generation of free radicals, in MCF-7 cells. N-acetyl cysteine was utilized as a positive (protective) control. Figure 6 indicates that sildenafil was, in fact, partially protective against hydrogen peroxide in the breast tumor cell. This observation indicates that adriamycin is unlikely to be acting through a free radical mediated mechanism in the tumor cells; otherwise, we might have expected at least a partial protective effect with sildenafil. Interestingly, however, in Figure 7, where free radical generation induced by hydrogen peroxide was directly monitored by a 2',7'-dichlorodihydrofluorescein (DCF) staining assay (6), we were unable to show an effect of sildenafil on free radical generation by hydrogen peroxide although N-acetyl cysteine clearly suppressed the free radical formation.

In order to more directly compare the response to sildenafil in both breast tumor and cardiac cells, we initiated studies in the H9c2 cardiomyocyte cell line. This is an embryonic cell line that replicates in culture and which has been used by a number of investigators as a model of cardiac function (7). Figures 8 indicates that in our initial studies sildenafil failed to provide a protective effect when these cells were treated with adriamycin in a cell viability assay. Figure 9 indicates that sildenafil was unable to protect the cardiomyocytes against adriamycin-induced apoptosis. Finally, Figure 10 demonstrates that sildenafil did not protect the cardiomyocytes against other chemotherapeutic drugs or ionizing radiation.

It is possible that the cardioprotection that was observed in previous studies is specific to adriamycin and therefore the lack of protection for other drugs may not be unexpected. However, the lack of protection against adriamycin is of concern relating to the potential clinical utility of sildenafil in the treatment of breast cancer. One likely explanation is that the current studies were performed with exposure of the cardiomyocytes to high concentrations of adriamycin for an extended period of time, which does not simulate the clinical conditions, where circulating drug levels are elevated for only 2-4 hours. We are currently repeating these experiments at more clinically relevant doses of adriamycin in order to determine whether protection by sildenafil will be detected.

Another possible explanation relates to the source of the cardiomyocytes. The previous reports from Dr. Kukreja's group utilized primary myocytes (1), while our studies utilized an embryonic cardiac cell line that can reproduce. It is possible that the response to sildenafil will differ based on the experimental model system utilized. We are examining specific elements, such as the levels of phosphodiesterase-5 expression, to determine the factors that could be involved in the putative protective effects of sildenafil.

KEY RESEARCH ACCOMPLISHMENTS

1. Verified lack of protection of various breast tumor cell lines from adriamycin by sildenafil.

2. Demonstrated lack of protection of breast tumor cells by sildenafil from various antitumor drugs and ionizing radiation.

REPORTABLE OUTCOMES

None at the present time.

CONCLUSIONS

Sildenafil fails to protect breast tumor cells from adriamycin (doxorubicin) in terms of growth inhibition as well as suppression of clonogenic survival.

Sildenafil fails to protect breast tumor cells against the actions of other conventional chemotherapeutic drugs or ionizing radiation.

The action of sildenafil in the breast tumor cell is unlikely to be occurring through the generation of free radicals.

Sildenafil does not appear to provide protection against adriamycin toxicity in a cardiomyocyte model. However, this may relate to the extreme exposure conditions of drug concentration and time. Alternatively, this model system may not express the target enzyme for sildenafil, phosphodiesterase-5. Our current studies are designed to address the limitations of this experimental model and to identify the conditions whereby sildenafil is cytoprotective in the heart.

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SUPPORTING DATA

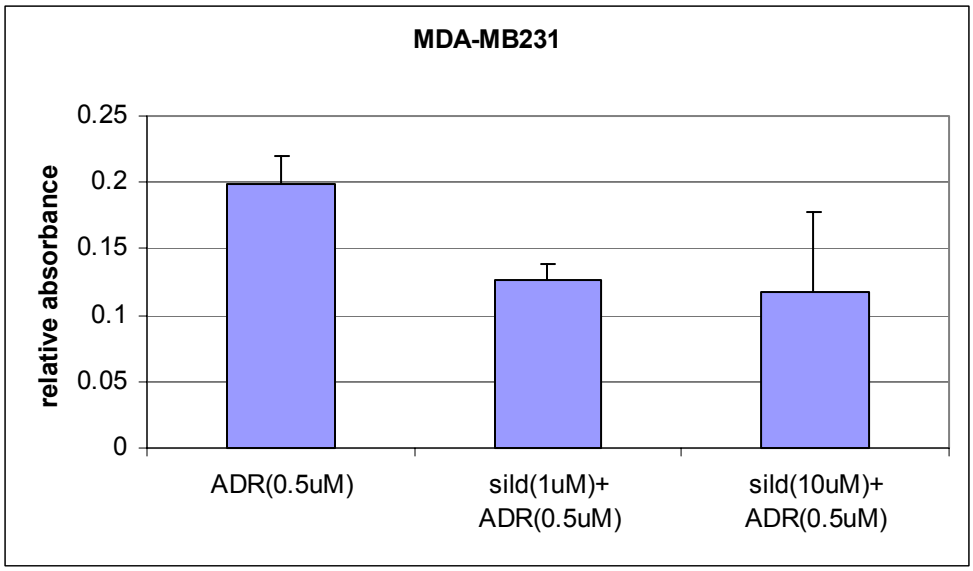


Figure 1: Lack of protection of MDA-MB231 breast tumor cells from adriamycin by sildenafil. Cells were exposed to sildenafil (10μM) for 1 hour prior to incubation with adriamycin (0.5μM) for 72 hrs. Relative absorbance was measured by the MTT assay and indicates the number of viable cells. Absorbance of untreated controls was in the range of 0.6-0.8.

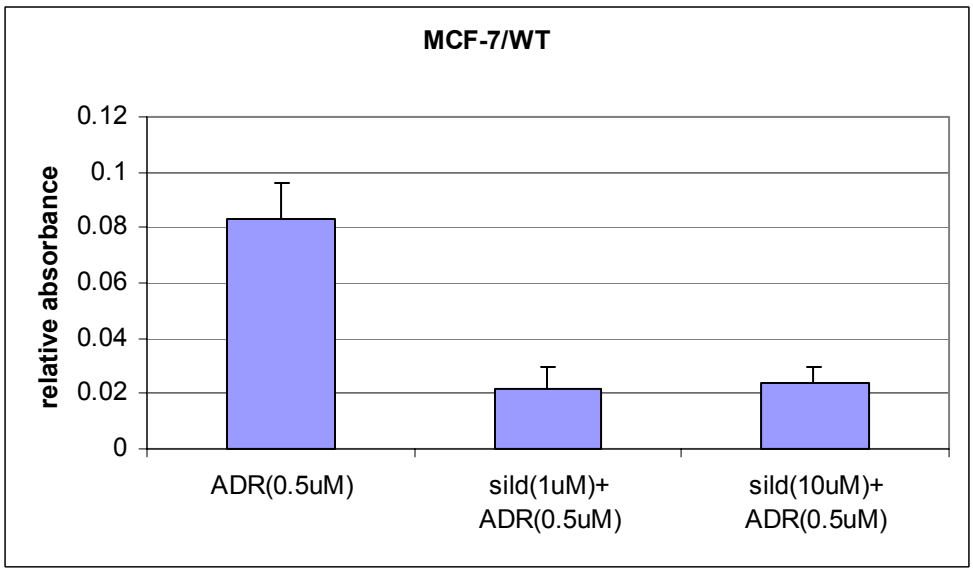


Figure 2: Lack of protection of MCF-7 breast tumor cells from adriamycin by sildenafil. Cells were exposed to sildenafil (10μM) for 1 hour prior to incubation with adriamycin (0.5μM) for 72 hrs. Relative absorbance was measured by the MTT dye assay and indicates the number of viable cells. Absorbance of untreated controls was in the range of 0.5-0.6.

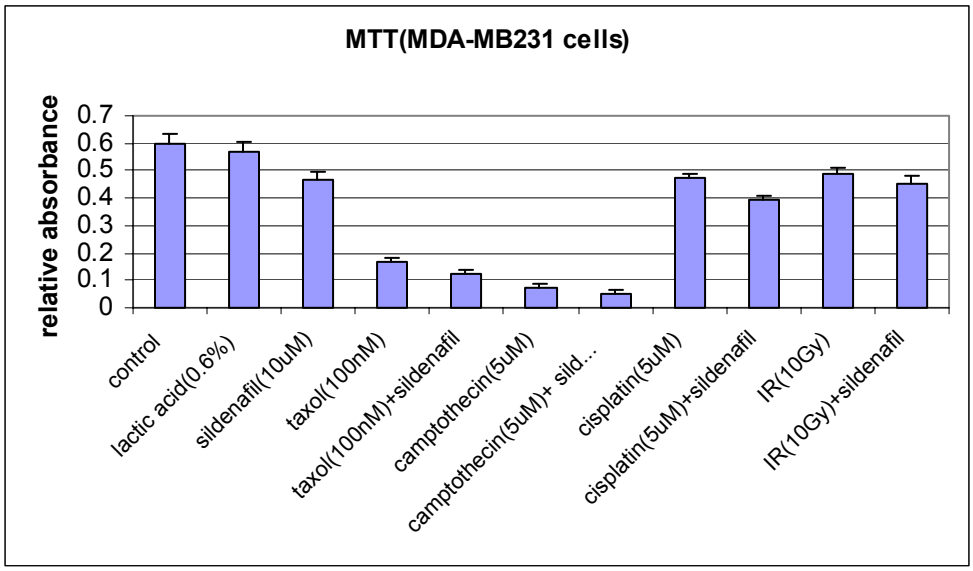


Figure 3. Lack of protection of MDA-MB231 breast tumor cells from drugs and radiation by sildenafil. Cells were incubated with sildenafil (10μM) followed by various antitumor drugs at the indicated concentrations or by 10 Gy of irradiation. Relative absorbance was measured by the MTT assay and indicates the number of viable cells.

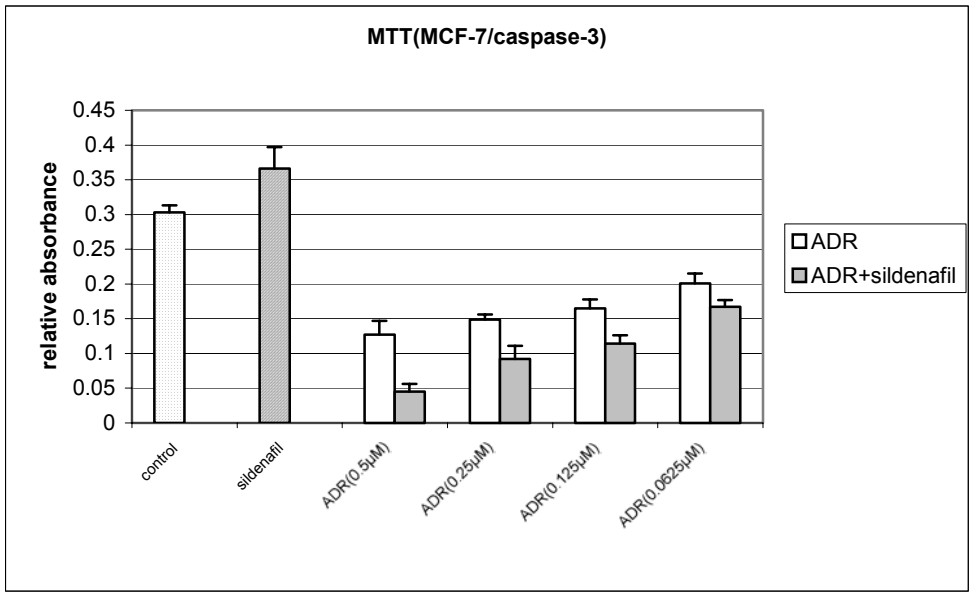


Figure 4: Lack of protection of MCF-7/caspase 3 breast tumor cells from adriamycin by sildenafil. Cells were exposed to sildenafil (10μM) for 1 hour prior to incubation with various concentrations of adriamycin for 72 hrs. Relative absorbance was measured by the MTT assay and indicates the number of viable cells.

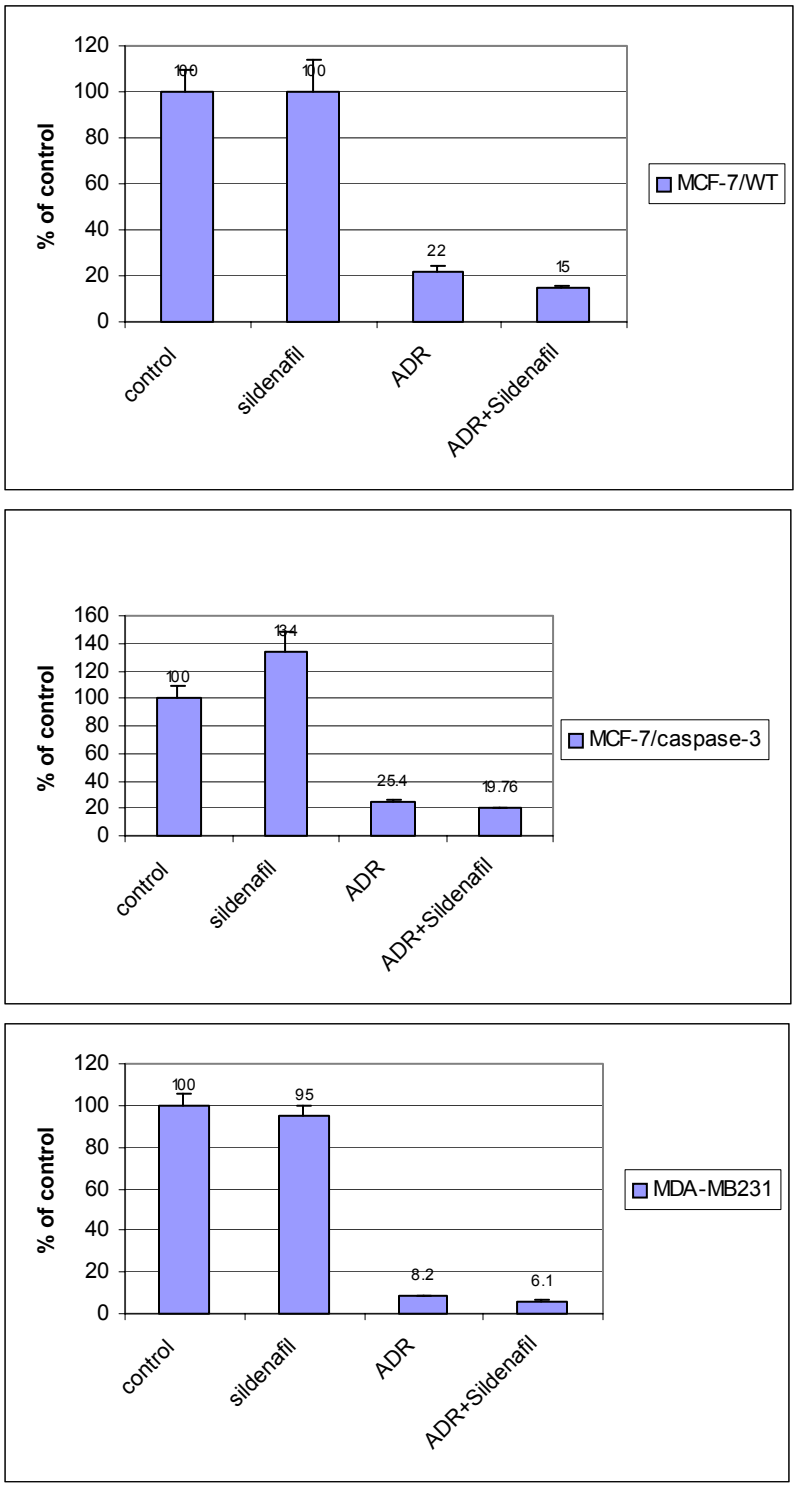


Figure 5. Lack of protection of various breast tumor cell lines by sildenafil from cytotoxic effects of adriamycin. Cells were exposed to 10μM sildenafil for 1 hour followed by 0.75μM Adriamycin for 2 hours. For control and sildenafil alone studies, 200 cells were plated. For studies with adriamycin, $1x10^4$ cells were plated. Colony numbers were determined after 14 days by clonogenic assay.

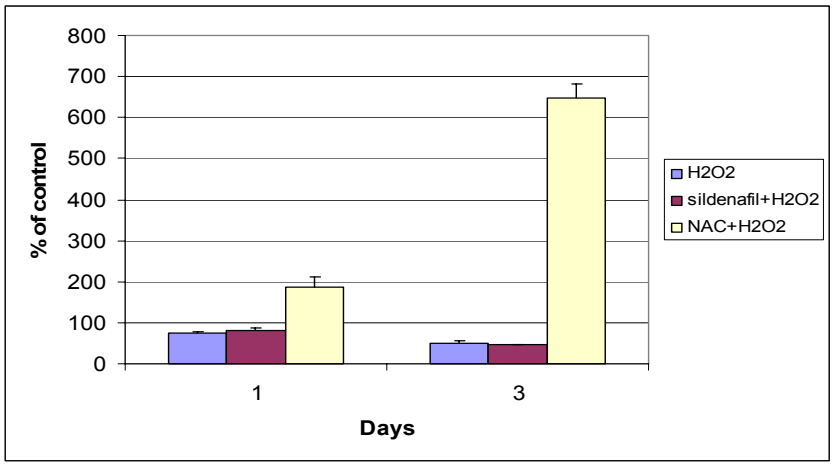


Figure 6. Influence of sildenafil and N-acetyl cysteine on sensitivity to hydrogen peroxide in MCF-7 wild type cells. Cells were exposed to 10µM sildenafil for 1 hour or 20mM NAC for 3 hours before treatment with 200µM H_2O_2 for 1hour. The sildenafil and NAC were maintained in the medium throughout the time of exposure to H_2O_2 and the one-day or 3 days afterwards. % of control represents viable cell number as measured by the trypan blue exclusion assay.

DCF staining for MCF-7/WT cells treated with hydrogen peroxide and sildenafil

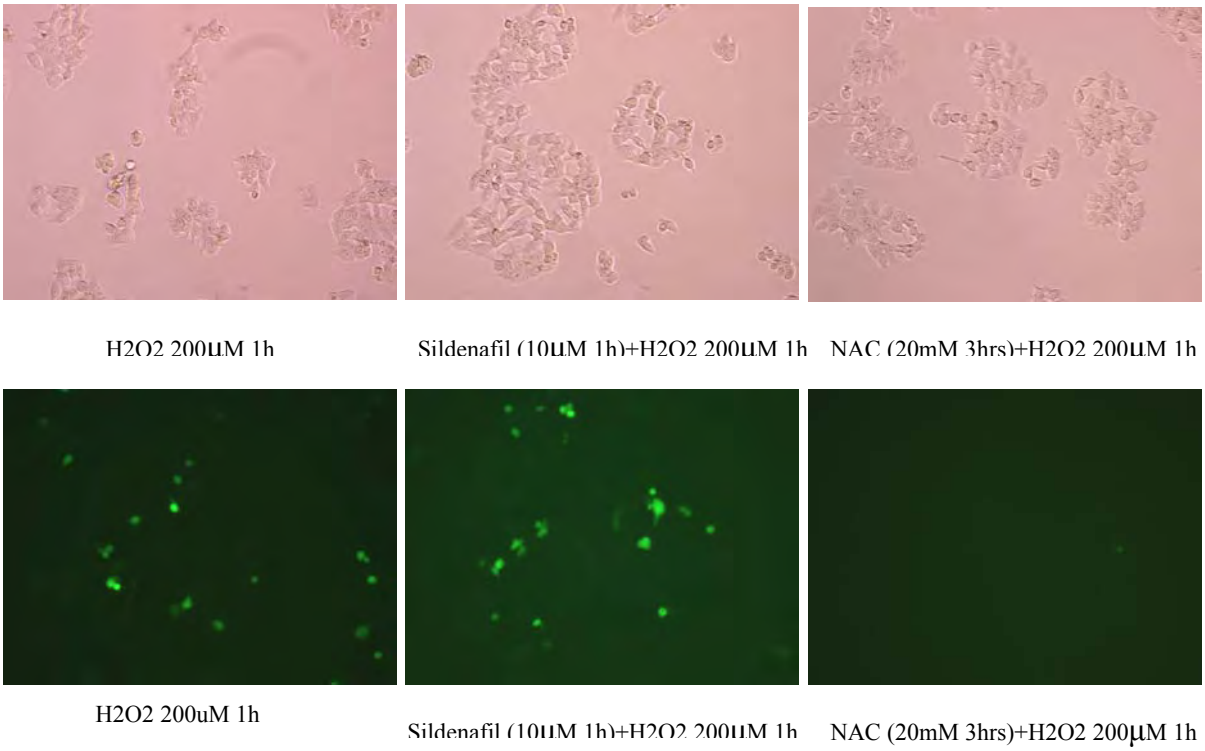


Figure 7: Influence of sildenafil and NAC on free radical generation by hydrogen peroxide in MCF-7 cells. Cells were exposed to 10µM sildenafil for 1 hour or to 20mM NAC for 3 hours prior to 100µM hydrogen peroxide for 1 hour. 2', 7'-dichlorodihydrofluorescein (DCF) staining was performed 24 hrs later.

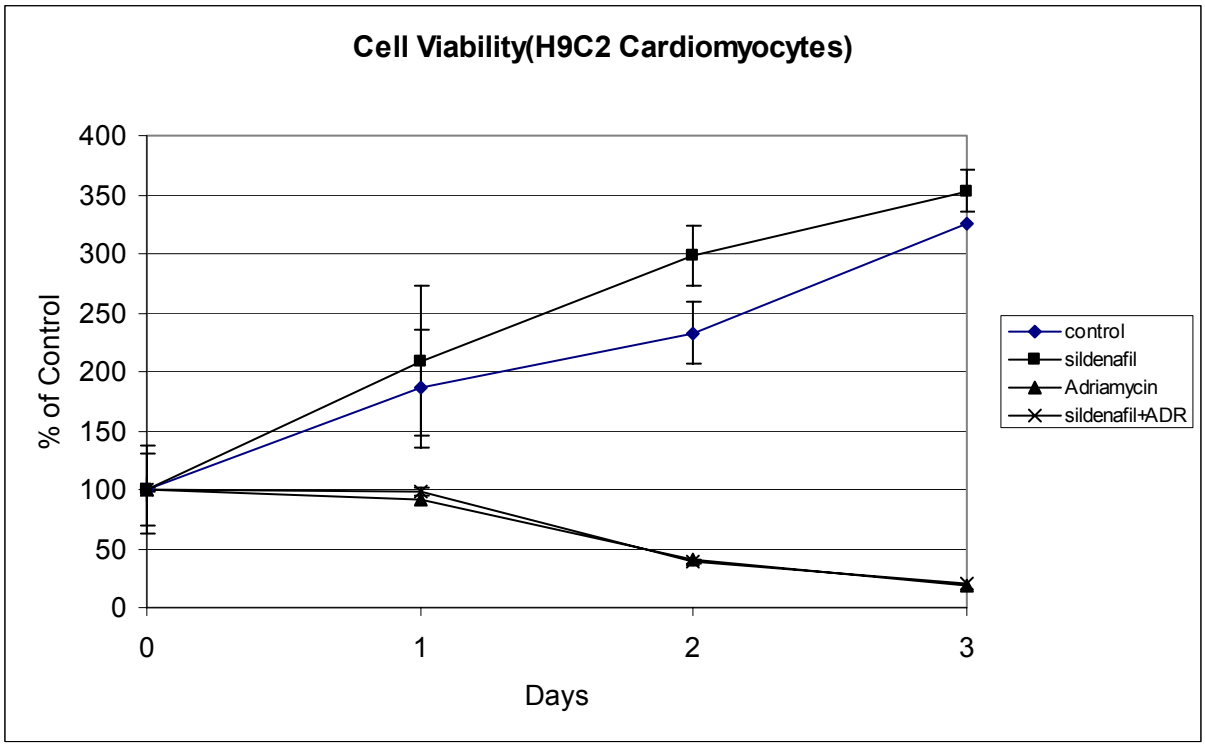
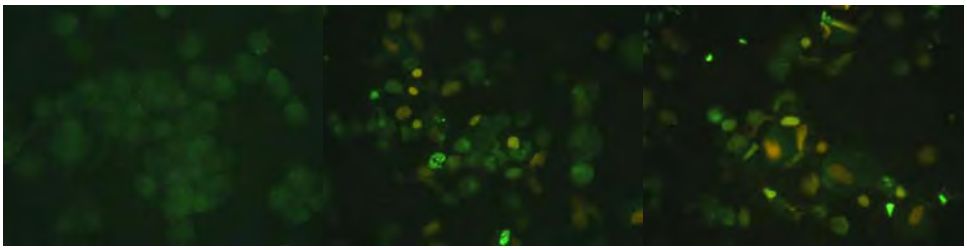


Figure 8. Inability of sildenafil to protect cardiomyocytes against the toxic effects of adriamycin. Cardiomyocytes were exposed to sildenafil (10μM for 1 hour) prior to exposure to 1 μM adriamycin for the indicated times; cell viability was monitored by trypan blue exclusion for a period of 3 days.



Sildenafil Adriamycin Sildenafil + adriamycin

Figure 9. Inability of sildenafil to protect cardiomyocytes against the toxic effects of adriamycin. Cardiomyocytes were exposed to sildenafil (10μM for 1 hour) prior to 1μM adriamycin. Apoptosis was monitored by the TUNEL assay for a period of 3 days. Results shown are from Day 3.

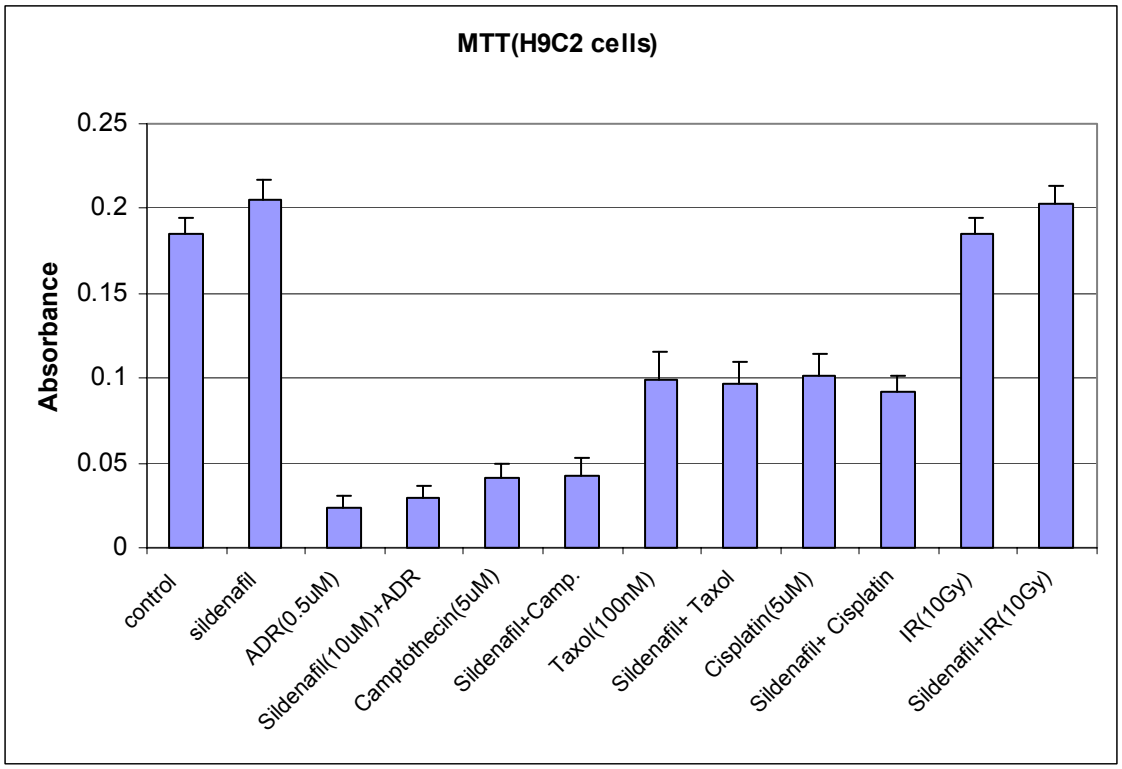


Figure 10. Inability of sildenafil to protect cardiomyocytes against the toxic effects of adriamycin. Cardiomyocytes were exposed to sildenafil (10μM for 1hour) prior to the chemotherapeutic drugs indicated or ionizing radiation. Absorbance reflects viable cell number after a period of 72 hrs based on the MTT dye assay.